International Journal of Food Microbiology 138 (2010) 189–199

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Review

What does genetic diversity of *Aspergillus flavus* tell us about *Aspergillus oryzae*?

Perng-Kuang Chang *, Kenneth C. Ehrlich

Southern Regional Research Center, Agricultural Research Service, U. S. Department of Agriculture, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70124, United States

ARTICLE INFO

Article history:
Received 3 September 2009
Received in revised form 21 January 2010
Accepted 24 January 2010

Keywords:
Aspergillus flavus
Aspergillus oryzae
Genomics
Gene cluster
Genetic diversity
Evolution

ABSTRACT

Aspergillus flavus and *Aspergillus oryzae* belong to *Aspergillus* section *Flavi*. They are closely related and are of significant economic importance. The former species has the ability to produce harmful aflatoxins while the latter is widely used in food fermentation and industrial enzyme production. This review summarizes the current understanding of the similarity of the *A. flavus* and *A. oryzae* genomes, the genetic diversity in *A. flavus* and *A. oryzae* populations, the causes of this diversity, and the relatedness of nonaflatoxigenic *A. flavus* strains to *A. oryzae*.

Published by Elsevier B.V.

Contents

1. Introduction

The genus *Aspergillus* represents groups of a very large number of asexual fungi (Fungi Imperfecti or Deuteromycetes) that are found in a broad range of habitats. Separation of individual species into various groups or sections was originally based on overlapping morphological or physiological characteristics (Raper and Fennell, 1965). *Aspergillus oryzae* and *Aspergillus flavus* belong to *Aspergillus* section *Flavi*. As with other fungi, these two species were conventionally distinguished from each other by morphological and cultural characteristics instead of by physiological or genetic traits (Jorgensen, 2007). *A. oryzae* has been widely used as the starter culture for the preparation of koji in the production of traditional Oriental fermented foods and alcoholic beverages, for example, soy sauce, miso, sake, and shochu. *A. oryzae* has also been an important source of many enzymes, such as glucoamylase, alpha-amylases and proteases used for starch processing, baking, and brewing worldwide (Machida et al., 2008). About two thirds of the bread production in the United States uses *A. oryzae* protease to release amino acids and peptides for yeast growth and gas production (Bigelis, 1992). The patented production of *A. oryzae* Taka-

* Corresponding author. Tel.: +1 504 286 4208; fax: +1 504 286 4419.
E-mail address: perngkuang.chang@ars.usda.gov (P.-K. Chang).

0168-1605/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2010.01.033

diastase, a neutral alpha-amylase, as a medicine in 1894 marked the beginning of modern enzyme biotechnology (Bennett, 1985). *A. oryzae* is capable of expressing high levels of heterologous enzymes. This ability led to the commercial production in *A. oryzae* of a recombinant lipase for use in detergents in 1988 by Novo Nordisk in Japan (Barbesgaard et al., 1992). *A. oryzae* and its fermentation byproducts are also used as probiotic and feed supplements for livestock (Lee et al., 2006b).

A. flavus, classified as a separate species but genetically almost identical to *A. oryzae*, is not used for commercial applications mainly due to its capability of producing aflatoxins (Scheidegger and Payne, 2003). *A. flavus* is thought to be predominately a saprophyte that grows on dead plant and animal tissue in the soil. Of all *Aspergillus*, it is the one most associated with preharvest contamination of certain crops. Because of its small spores and its ability to grow at 37 °C it can also be pathogenic to animals and humans. Infection by *A. flavus* has become the second leading cause of human aspergillosis (Krishnan et al., 2009). *A. flavus* can infect corn, peanuts, cotton, and nut trees as well as other crops and growth on these agricultural commodities often leads to contamination with aflatoxin B_1, a toxic and potent carcinogenic compound. Some researchers believe that *A. oryzae* is widely distributed in nature while others think that *A. oryzae* strains are just variants of *A. flavus* that have been domesticated through years of selection under artificial production environments. *A. oryzae* strains intended for commercial use commonly exhibit sparse sporulation, have floccose aerial mycelia and produce few or no sclerotia. The lack of these characteristics could be detrimental to dissemination and survival of *A. oryzae* in the field. However, there is evidence that certain nonaflatoxigenic *A. flavus* isolates obtained from the field have characteristics of *A. oryzae*. Therefore, *A. oryzae* may be a morphotypic variant of typical *A. flavus*.

Because of economic and food safety issues, *A. oryzae* continues to be classified as a separate taxon from *A. flavus*. The long history of safe use of *A. oryzae* by the food fermentation industry and lack of aflatoxin production has earned it GRAS (generally recognized as safe) status. In the past two decades, molecular biological techniques have been used to distinguish species in the *Aspergillus* section *Flavi*. These include restriction fragment length polymorphism (Klich and Mullaney, 1987), amplified fragment length polymorphism (Montiel et al., 2003), hybridization with aflatoxin biosynthetic genes (Klich et al., 1995), analysis of ribosomal DNA internal transcribed spacer regions (Kumeda and Asao, 2001), and single nucleotide polymorphisms (Lee et al., 2006a). In general, these methods are able to distinguish the *A. flavus*/*A. oryzae* group from the *A. parasiticus*/*A. sojae* group but do not separate *A. flavus* from *A. oryzae*. The goal of unambiguously distinguishing *A. oryzae* from *A. flavus* as a distinct species has not been realized. The unsuccessful endeavors further indicate the close relatedness of the two species.

A. flavus is not a monophyletic species. A morphological variant population has been identified. This variant, previously called *A. flavus* var *parvisclerotigenus* (Saito and Tsuruta, 1993) and distinguished by sclerotial size from "typical" *A. flavus*, has been reclassified as a separate species by Frisvad et al. (2005). Sclerotia are hardened masses of mycelia that serve as over-winter reproductive forms and remain dormant in soil until conditions are favorable for growth. The typical *A. flavus* isolates are called the L-strain whose average sclerotial size is greater than 400 μm while isolates of the variant strain called the S-strain have sclerotial size less than 400 μm (Cotty, 1989). Sclerotium morphology is a poor indicator of phylogeny. On laboratory growth media, when grown in the dark, S-strain isolates produce higher levels of aflatoxins, more abundant sclerotia, and fewer conidia than L-strain isolates. Like *A. oryzae*, attempts to distinguish S-strain *A. flavus* from typical *A. flavus* have not been accepted by all taxonomists. Nonaflatoxigenic S-strain isolates are very rarely found in natural environments or as crop contaminants (Orum et al., 1997).

Other species have been classified within section *Flavi* and five of these were found to produce aflatoxins. Two new species, *A. minisclerotigenes* and *A. arachidicola,* have been identified that produce both B- and G-aflatoxins but have sclerotia and conidia that resemble those of S-strain *A. flavus* (Pildain et al., 2008). Also some species within section *Nidulantes* and *Versicolores* make aflatoxin precursors and aflatoxins (Cary et al., 2009). Why some species are able to produce aflatoxins while others do not is not well understood and this distinction has important implications for human health and food safety.

2. Comparison of the *A. oryzae* and *A. flavus* genomes to those of other aspergilli

In December 2005, a consortium in Japan consisting of universities, institutions and the brewing industry led by The National Institute of Advanced Industrial Science and Technology released the genome sequence of *A. oryzae* RIB40 (ATCC 42149) (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao). Later the genome sequence of *A. flavus* NRRL3357 was released by The Institute for Genomic Research (TIGR, Rockville, Maryland, USA, now J. Craig Venter Institute, JCVI) with funding from the Microbial Genome Sequencing Project to scientists at North Carolina State University (http://www.aspergillusflavus.org/genomics/). Together with studies on various aspects of the genetics of *A. oryzae* and *A. flavus*, these genome sequences have provided a wealth of information with regard to evolution and stability of the aflatoxin gene cluster as well as an assessment of the abilities of isolates of the same species and of different *Aspergillus* species to undergo recombination.

The assembled *A. oryzae* genome is about 37 Mb and organized in eight chromosomes. It is predicted to encode 12,074 proteins (Machida et al., 2005). The genome size is comparable to that of the closely related *A. flavus* NRRL3357, which is also about 37 Mb and consists of eight chromosomes (Payne et al., 2006). A comparative analysis of *A. oryzae* and *A. flavus* genomes revealed striking similarity between them. An array based genome comparison found only 43 genes unique to *A. flavus* and 129 genes unique to *A. oryzae*. Only 709 genes were identified as uniquely polymorphic between the two species (Georgianna and Payne, 2009).

To date, all other species in the genus *Aspergillus* whose genomes have been sequenced have eight chromosomes, but their genome sizes are smaller. The genome size of *A. fumigatus* is about 30 Mb, *A. nidulans* 31 Mb, *A. niger* 34–35 Mb, *A. terreus* 35 Mb and *A. clavatus* 35 Mb (NCBI Genome Projects, http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj). Comparison of the genome sequences of *A. fumigatus*, *A. nidulans*, and *A. oryzae* showed that the genomes of *A. fumigatus* and *A. nidulans* are predicted to encode 1412 and 2444 fewer proteins than the *A. oryzae* genome, respectively. Excluding singletons (genes without homologs in the fungal genome databases) the *A. oryzae* genome contains 16 and 26% more genes than the two species, respectively (Galagan et al., 2005). Syntenic analysis of the three aspergilli showed that common syntenic blocks and specific blocks are organized in a mosaic manner in the *A. oryzae* genome. Phylogenetic analysis with whole-genome data indicates that *A. nidulans* branched off from a common ancestor earlier than *A. oryzae* and *A. fumigatus*. Thus, the increase in the *A. oryzae* genome size likely is due to lineage-specific sequence expansion rather than loss of sequence in the *A. nidulans* and *A. fumigatus* genomes.

3. Origins of the extra genes in *A. oryzae* and *A. flavus* genomes

Machida et al. (2005) noticed that some genes from these non-syntenic blocks are paralogs of other orthologous genes that have a syntenic relationship among the aforementioned three aspergilli. In contrast to orthologs, which are genes from a common ancestor and encode proteins with the same function in different species, paralogs are

genes evolved by duplication and encode proteins with similar but not identical functions. These paralogous genes in *A. oryzae* are probably not from recent gene duplications but are products of much older divergence events. Khaldi and Wolfe (2008) examined the origins of these extra genes in *A. oryzae* and found no evidence of whole-genome or segmental duplication. They identified 456 gene pairs that are paralogous in *A. oryzae* but are only single-copy in *A. nidulans* and *A. fumigatus*. This number accounts for 19% of all paralogous gene pairs of *A. oryzae* which is significantly higher than those calculated from *A. nidulans* (12%) and *A. fumigatus* (11%). Since independent, parallel loss of the paralogs in *A. nidulans* and *A. fumigatus* seems to be unlikely, horizontal gene transfer has been considered a cause for the genome increase in *A. oryzae*. This mechanism, however, can only explain the possible origins of about one-third of the paralogs, which are from *Sordariomycetes* or other *Aspergillus* species (*A. niger*, *A. terreus*, *A. clavatus*), whose genomes are 13 to 16% larger than those of *A. fumigatus* and *A. nidulans*. This increase is estimated to represent about 1000 to 1300 more genes. The *A. niger* genome sequence and those of other aspergilli have a high level of synteny. The *A. niger* ATCC 1015 sequence contains 11,197 genes (DOE Joint Genome Institute, http://genome.jgi-psf.org/Aspni5/Aspni5.home.html). Another species, *A. clavatus*, is evolutionarily closer to *A. fumigatus* than to *A. terreus*. The unique increase in genome size found in *A. oryzae* and *A. flavus* compared to these related *Aspergillus* species is reflective of an as yet not well understood adaptive response to a new niche that developed at the time of separation estimated to be about 5 to 8 Mya (see below).

4. Genetic features common to *A. oryzae* and *A. flavus*

Rokas et al. (2007) conducted intraspecies genome comparisons of *A. niger* strains, *A. fumigatus* strains, and *A. oryzae* and *A. flavus*. The degrees of identity at the genome, gene and protein levels between *A. oryzae* and *A. flavus* resemble those from the comparison of the *A. fumigatus* strains and of the *A. niger* strains. They concluded that *A. oryzae* is not a distinct species. This finding is consistent with an earlier DNA reassociation study (Kurtzman et al., 1986) which showed 100% complementarity between *A. oryzae* and *A. flavus*. Nikkuni et al. (1998) also showed that the 18S rDNA (1733 nt) and 5.8S rDNA (157 nt) of *A. flavus* and *A. oryzae* isolates are identical.

In addition to the conserved genetic features, unique repeat sequences in telomeres and retrotransposons also have revealed the close relatedness of *A. oryzae* and *A. flavus*. Telomeres are nucleoprotein complexes located at the ends of chromosomes. These specialized structures consist of tandem nucleotide repeats and chromatin proteins whose functions are not completely understood. Telomeres protect the linear termini of chromosomes against degradation during cell division. The telomeric repeat sequence, TTAGGG, is the common sequence for many filamentous fungi (Table 1), animals and humans although this repeat is modified to TTAGG in insects (Vitkova et al., 2005) and TTTAGGG in plants (Fuchs et al., 1995). In *A. fumigatus* the TTAGGG telomeric repeat has been identified at both ends of its eight chromosomes. Kusumoto et al. (2003) first reported that *A. oryzae* has a unique dodeca-nucleotide telomeric repeat sequence, TTAGGGTCAACA. Except for the telomere at one end of chromosome 8, this telomeric repeat sequence is at the ends of the chromosomes of *A. oryzae* RIB40. The same telomeric repeat sequence also has been identified from one end of chromosome 3 in many *A. flavus* isolates that contain various deletions in the subtelomeric region (Chang et al., 2005).

Retrotransposons are retrovirus-like DNA elements present in eukaryotic genomes. They replicate and insert into new chromosomal locations via RNA intermediates. McAlpin and Mannarelli (1995) constructed a probe named pAF28, which contains a putative repetitive DNA sequence isolated from *A. flavus* NRRL6541, for genotyping. This probe showed stronger hybridization signals to *A. flavus* and *A. oryzae* than to others species in *Aspergillus* section *Flavi*. It has been used to distinguish *A. flavus* isolates belonging to different vegetative compatibility groups (Wicklow et al., 1998; McAlpin et al., 2002), to track clinical and environmental sources of *A. flavus* isolates (James et al., 2000) and to study genetic diversity of *A. oryzae* strains (Wicklow et al., 2002; Wicklow et al., 2007). Okubara et al. (2003) sequenced the 6.2-kb DNA fragment insert in pAF28 and found that the insert contains a 4.5-kb region that encodes a truncated retrotransposon. Hua et al. (2007) subsequently used a novel PCR strategy to clone the missing region of the retrotransposon from the genomic DNA of *A. flavus* NRRL6541. The compiled retrotransposon sequence was named *AFLAV*. It has two flanking long terminal repeats (LTRs) and between the LTRs is the 7.8-kb retrotransposon encoding capsid (Gag) and polyprotein (Pol). The order of the catalytic domains in Pol places *AFLAV* in the *Tf1/sushi* subgroup of the *Ty3/gypsy* retrotransposon family. The *A. flavus* NRRL3357 genome contains only short stretches of DNA resembling the LTRs of *AFLAV* but no region similar to the *gag* and *pol* genes of *AFLAV*. A survey of more than 50 *A. flavus* strains collected from different locations in the United States indicates that many strains containing *AFLAV* have extensive deletions in the regions encoding Gag and Pol. Interestingly, *A. oryzae* RIB40 genome contains two highly homologous copies of *AFLAV* (Hua et al., 2007). They are located on chromosome 1 and are about 550 kb apart. The two *A. oryzae* retrotransposons differ only in one nucleotide and have 94% nucleotide identity to *AFLAV*. The predicted amino acid sequences of Gag and Pol of the *A. oryzae* retrotransposons also have 94% identity to those encoded by *AFLAV*. The degrees of identity at the nucleotide and amino acid levels are comparable to those found among some groups of *A. oryzae* strains (our unpublished results).

5. The phylogenetic relationship of *A. flavus* and *A. oryzae*

Before the advent of the genomics era, the genetic relationship between *A. flavus* and *A. oryzae* was mainly inferred from available genes, especially those associated with aflatoxin biosynthesis. Geiser et al. (1998) utilized the multi-locus genotypes approach, based on restriction site polymorphisms of 11 genes defined by the presence (allele 1) or absence (allele 0) of each locus, to provide the first examination of the phylogeny of 31 Australian *A. flavus* strains and 5 *A. oryzae* strains. The *A. flavus* strains consisted of 16 different genotypes, and phylogenetic analysis indicated that they fell into two groups (I and II). Four *A. oryzae* strains and one *A. oryzae* were found to be identical to *A. flavus* genotype C and genotype Q of group I,

Table 1
Telomeric repeat sequences of fungi.

Species	Telomeric repeat	Reference
Aspergillus clavatus	TTAGGG	Broad Institute[a]
Aspergillus flavus	TTAGGGTCAACA	Chang et al., 2005
Aspergillus fumigatus	TTAGGG	Broad Institute
Aspergillus nidulans	TTAGGG	Bhattacharyya and Blackburn, 1997
Aspergillus oryzae	TTAGGGTCAACA	Kusumoto et al., 2003
Botrytis cinerea	TTAGGG	Levis et al., 1997
Cladosporium fulvum	TTAGGG	Coleman et al., 1993
Coccidioides immitis	TTAGGG	Broad Institute
Coccidioides posadasii	TTAGGG	Broad Institute
Fusarium graminearum	TTAGGG	Broad Institute
Fusarium oxysporum	TTAGGG	Powell and Kistler, 1990
Glomus intraradices	TTAGGG	Hijri et al., 2007
Histoplasma capsulatum	TTAGGG	Broad Institute
Magnaporthe grisea	TTAGGG	Farman and Leong, 1995
Neurospora crassa	TTAGGG	Schechtman, 1990
Pestalotiopsis microspora	TTAGGG	Long et al., 1998
Pneumocystis carinii	TTAGGG	Underwood et al., 1996
Podospora anserina	TTAGGG	Javerzat et al., 1993
Sclerotinia sclerotiorum	TTAGGG	Broad Institute
Ustilago maydis	TTAGGG	Guzman and Sanchez, 1994

[a] From the fungal genome database at Broad Institute, http://www.broadinstitute.org/science/data#.

respectively. The *omtA* gene is an aflatoxin biosynthetic gene, which encodes an *O*-methyltransferase required for the conversion of sterigmatocystin to *O*-methylsterigmatocystin and dihydrosterigmatocystin to dihydro-*O*-methylsterigmatocystin (Yu et al., 2004). In a related study, Geiser et al. (2000) compared partial *omtA* sequences (*omt12*) of 33 S- and L-strain *A. flavus* and three *A. oryzae* isolates. They showed that three well-supported clades within group I (IA, IB and IC) could be distinguished. Subgroup IB, a monophyletic *A. flavus* clade that exclusively consisted of L-strain isolates, also included three *A. oryzae* isolates collected from California.

All toxigenic *A. flavus* isolates produce B aflatoxins, but *A. parasiticus* isolates produce both B and G aflatoxins. The lack of G aflatoxin production by *A. flavus* results from deletions in the *norB-cypA* intergenic region in the aflatoxin gene cluster. The *cypA* gene is necessary for the synthesis of the cytochrome P450 monooxygenase, CypA, which is required for G aflatoxin formation (Ehrlich et al., 2004). Two types of deletions are found in the *norB-cypA* region of *A. flavus* isolates when the sequence in this region is compared to that of *A. parasiticus*. Type I deletion (1.5 kb) overlaps mostly with type II deletion (1.0 kb). Type II deletion, even though smaller, actually is comprised of two separate deleted regions; one not found in type I deletion is a 32-bp deletion in the *norB* gene encoding amino acid residues 300–310 of the NorB aryl alcohol dehydrogenase. Therefore, the type I and type II deletions arise from independent DNA losses in the *norB-cypA* region. Chang et al. (2006), included the two unique deletion patterns, and other genotypic, phenotypic, and chemotypic criterion, in phylogenetic analyses of 29 North American *A. flavus* strains and 6 *A. oryzae* strains from different regions of the world. As reported by Geiser et al. (2000), *A. flavus* isolates form a polyphyletic assemblage. Similarly, the *A. oryzae* isolates also formed a polyphyletic assemblage. Four of the six *A. oryzae* strains group in a clade together with a number of nonaflatoxigenic L-strain *A. flavus* isolates from various regions in the United States. The aflatoxigenic S-strain isolates and aflatoxigenic L-strain isolates contain type I and type II *norB-cypA* deletions, respectively. Although two of the *A. oryzae* isolates are missing the entire *norB-cypA* region, the other four *A. oryzae* isolates contain only a type I deletion, suggesting that a nonaflatoxigenic L-strain isolate with the type I deletion is a common ancestor of these isolates. However, none of the six *A. oryzae* isolates is cladally related to aflatoxigenic S-strain *A. flavus* isolates. Therefore, the most recent common ancestor of these *A. oryzae* isolates may be a subgroup of nonaflatoxigenic L-strain *A. flavus*, which might have diverged from the aflatoxigenic S-strain *A. flavus*.

6. Genetic diversity of *A. oryzae* groups

Kusumoto et al. (2000) first observed large deletions in the aflatoxin gene cluster in many of the *A. oryzae* strains used for food fermentation. The *A. oryzae* strains could be separated into groups depending on how much of the aflatoxin cluster was still present. Group 1 has the *pksA*, *fas1A*, *aflR*, *ver1* and *vbs* orthologs, group 2 has the *ver1* and *vbs* orthologs, and group 3 has the *vbs* ortholog (see Fig. 1). The seemingly sequential loss of the aflatoxin gene homologs in *A. oryzae* suggests a unidirectional deletion of portions of the aflatoxin gene cluster. Tominaga et al. (2006) later examined 210 *A. oryzae* RIB (National Research Institute of Brewing) strains for *aflT*, *nor1*, *aflR*, *norA*, *avnA*, *verB* and *vbs* and confirmed that the previously classified three groups define almost all of the deletions in *A. oryzae* species. They found that more than half of the RIB strains belong to group 1 (122 strains, 58.1%), which includes the type strain RIB1301 and the strain, RIB40, used in genome sequencing. Comparison of predicted amino acid sequences of the aflatoxin biosynthetic enzymes and regulatory proteins of *A. oryzae* RIB40 and S-strain *A. flavus* AF70 show that they share 97 to 99% identity except for AflT, NorA, and VerA, which have 93% or lower amino acid identity. At the nucleotide level, a 257-bp deletion in the *aflT* region, a frameshift mutation in *norA*, and a base substitution in *verA* were found in *A. oryzae* RIB40. Seventy-seven of the *A. oryzae* RIB strains (36.7%) belong to group 2. Using RIB62, an isolate obtained from sake-koji in 1953, as the representative strain, Lee et al. (2006c) analyzed the region flanking the partial aflatoxin gene cluster from other RIB strains in this group. Their PCR amplification with location-specific primers, one common to RIB40 and RIB62 and the other specific to RIB62, showed that all group 2 strains have the same unique structure adjacent to the 'breakdown and restoration' region, which is located upstream of the *ver1* gene. This suggests a common origin for the group 2 *A. oryzae* strains. Although the entire *norB-cypA* region is missing in the group 2 strains, a comparison of a limited region in the remaining aflatoxin gene cluster of RIB62 (GenBank accession number: AB195804) to that of the S-strain *A. flavus* AF70 suggested that the group 2 strains evolved from S-strain *A. flavus* because they share 99% nucleotide identity. A comparison of the same region in RIB40 and AF70 showed only 92% nucleotide identity, which suggests further divergence of group 1 and group 2 *A. oryzae*. Group 3, which only contains the genes *verB* and *vbs* or *vbs* (4.3% of the RIB strains), and other strains (group 4, 0.9%), which appear to have lost almost all of the aflatoxin gene cluster, also were identified. Ogasawara et al. (2009) recently reported a novel active transposable element, *Crawler*, from four industrial strains of *A. oryzae* (OSI1013, RIB40, RIB128 and RIB209). It encodes a transposase of 357 amino acids, which is highly homologous to the transposase of *impala*, a *Tc1*/*mariner* superfamily class II DNA transposon from *Fusarium oxysporum*. Consistently, the *Crawler* transposon of group 1 *A. oryzae* RIB40 shares 90% nucleotide identity to that of an isolate from group 2 *A. oryzae* (RIB128) and also to an isolate of group 4 *A. oryzae* (RIB209). In contrast, the *Crawler* transposons of RIB128 and RIB209 share >99% nucleotide identity, which suggests that these isolates evolved from the same ancestor.

7. Genetic similarity of the aflatoxin gene cluster of *A. flavus* and *A. oryzae*

A recent survey by Criseo et al. (2001) showed that more than 60% of nonaflatoxigenic *A. flavus* isolates collected from food, feed and officinal plants in Italy contain deletions in the aflatoxin gene cluster. This finding is consistent with a study (Chang et al., 2005), which found that large deletions of the aflatoxin gene cluster are prevalent in non-toxigenic *A. flavus* isolates collected from soils in the southern United States. In that study, eight distinct deletion patterns, including one missing the entire aflatoxin gene cluster, were identified (Fig. 1). Patterns A and D correspond to the type I and II deletions in the *norB-cypA* region, respectively. Pattern B contains nucleotide deletions upstream of the *norB-cypA* region and in the hybrid polyketide and nonribosomal polyketide synthase gene necessary for cyclopiazonic acid biosynthesis (Chang et al., 2009). Pattern E, which includes two isolates from Texas (TX13-5 and TX21-5) has a breakpoint within the 5′ untranslated region (UTR) of the *ver1* gene. Identical sequence near *ver1* and upstream of the breakpoint is found in *A. oryzae* SRRC2098 (ATCC11493), an isolate from soybean-wheat flour mixture in Japan, and in SRRC2103 (ATCC10196), an isolate from pine board in USA. Tominaga et al. (2006) sequenced the entire region from the breakpoint to the telomere in RIB62 belonging to group 2 *A. oryzae*. The first 3.8 kb of this 7.8-kb region (GenBank accession number: AB176961) did not match known nucleotide sequence in either *A. flavus* NRRL3357 or *A. oryzae* RIB40 or with other sequences deposited in the databases (Fig. 2). However, BLASTX analysis of this sequence found that the first 0.5 to 1.5 kb probably encodes part of the AmdA positive regulator involved in acetamide degradation while the next 3.0 to 3.8-kb region probably encodes part of a cytochrome P450 monooxidase. The nucleotide sequence of the remaining 4.0 kb is 96% identical to a DNA segment in the subtelomeric region of *A. flavus* NRRL3357 (Fig. 2; also see Fig. 3 of the *ord1* to *ord2* region). Interestingly, we found that in *A. flavus* NRRL3357 an inverse

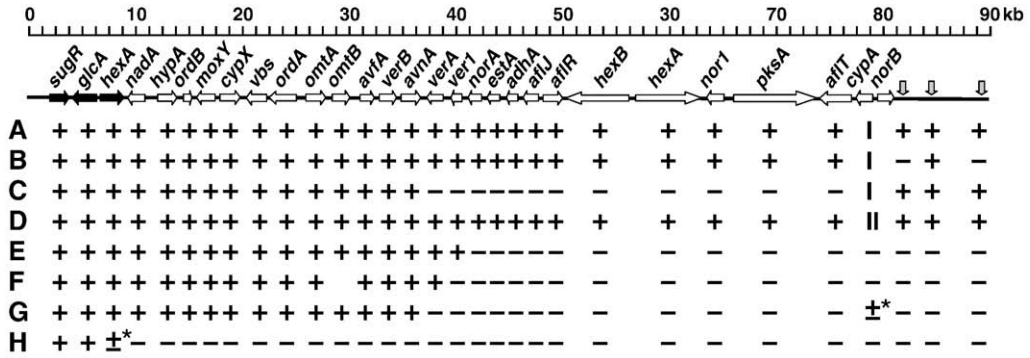

Fig. 1. Deletion patterns in the aflatoxin gene clusters of *A. flavus* isolates collected from agricultural soils of southern United States. I and II indicate type I and type II deletions in the *norB-cypA* region. The region containing *hexA-glcA-sugR* represents the region downstream of the aflatoxins cluster. Plus (+) and minus (−) indicates the presence or absence of a particular region while ±* indicates the presence of telomeric repeats at the specified region.

telomeric repeat (TAATGTTGACCCTAATGTTGACCCTAATGT) immediately precedes the beginning of the region corresponding to the last 4.0 kb in the 7.8-kb region of RIB62. This unique telomeric repeat could be a cause of chromosomal instability or translocations in RIB62. Sequence analysis of *A. flavus* TX13-5, *A. flavus* TX21-5, *A. oryzae* SRRC2098 and *A. oryzae* SRRC2103 revealed the same translocation junction as that of *A. oryzae* RIB62 (GenBank accession numbers: FJ865359–62). Further analyses of the subtelomeric region by PCR with location-specific primers derived from *A. flavus* NRRL3357 genome sequence suggest that *A. flavus* TX13-5 and TX21-5 retain 10 kb additional sequence beyond that leading to the telomere in RIB62. *A. oryzae* SRRC2098 and SRRC2103 and *A. flavus* NRRL3357 still have the entire region (Fig. 3). Therefore, group 2 *A. oryzae* isolates probably have the same common ancestor as the *A. flavus* isolates found in the field that have pattern E deletion. Most recently, Jiang et al. (2009) reported that the prospective biocontrol agent, *A. flavus* AF051, isolated from a peanut field in Jiangsu Province, China has the *A. oryzae* RIB62 genotype. Other *A. flavus* isolates having the same genotype are also present in various regions of China (Ma, personal communication) thereby revealing widespread geographical distribution of *A. flavus* isolates with this genotype. Although no *A. flavus* isolates with the group 3 deletion pattern of *A. oryzae* have been identified, the rare isolates with the group 4 *A. oryzae* deletion pattern reported by Tominaga et al. (2006) likely correspond to the *A. flavus* isolates missing the entire aflatoxin gene cluster from the United States and China (Chang et al., 2005; Yin et al., 2009). These results confirm that subtelomeric regions are regions of high genetic flux supporting increased levels of recombination, inversions, deletions, translocations and other genomic rearrangements that would be the nurturing grounds for the construction of new synthetic machinery under the constraint of co-regulation.

8. Forces driving the formation and loss of the aflatoxin gene cluster

Analyzing the ordering and clustering of genes in the aflatoxin pathway of five *Aspergillus* (*A. flavus*, *A. oryzae*, *A. parasiticus*, *A. fumigatus* and *A. terreus*) genomes, Carbone et al. (2007a) found that the aflatoxin genes were organized into seven distinct modules: *aflA*(*fas2*)/*aflB*(*fas1*), *aflR*/*aflS*(*aflJ*), *aflX*(*ordB*)/*aflY*(*hypA*), *aflF*(*norB*)/*aflE*(*norA*), *aflT*/*aflQ* (*ordA*), *aflC*(*pksA*)/*aflW*(*moxY*) and *aflG*(*avnA*)/*aflL*(*verB*). Since not all of the genes in these modules are contiguous across the *Aspergillus* species, it suggests that the cluster did not evolve by horizontal gene transfer. The evolution and retention of these modules may involve gene duplications where the gene copy retains the function of the pre-duplication gene, as observed with the aryl alcohol dehydrogenase genes, *aflF*/*aflE* (Cary et al., 1996) or the ferredoxin-fold oxidases, *hypB* and *hypC* (Ehrlich, unpublished results). Some genes such as *aflA* and *aflB* (Watanabe and Townsend, 2002) and *aflR* and *aflS* (Chang, 2003) are bidirectionally transcribed from a common promoter region. Common regulation would place selective pressure on retention of the modular organization of these genes in a gene cluster. Genes in other modules may encode proteins that form a coordinate enzyme pair for proper functionality, for example *aflX*/*aflY*, the genes required for the conversion of versicolorin A to demethylsterigmatocystin (Cary et al., 2006). However, genes organized in a module may become separated or lost. Lack of continuity of a module in one species but continuity in others could result from ancestral rearrangements leading to successful divergent lineages. Both *A. fumigatus* and *A. terreus* have partial clusters containing five genes: *aflC*, *aflS*, *aflR*, *aflX* and *aflY*. As in some *A. flavus* and *A. oryzae* the partial clusters in *A. fumigatus* and *A. terreus* may have resulted from gene losses that occurred in an ancestor with a more complete cluster. Alternatively, the ancestral cluster may have served a different function.

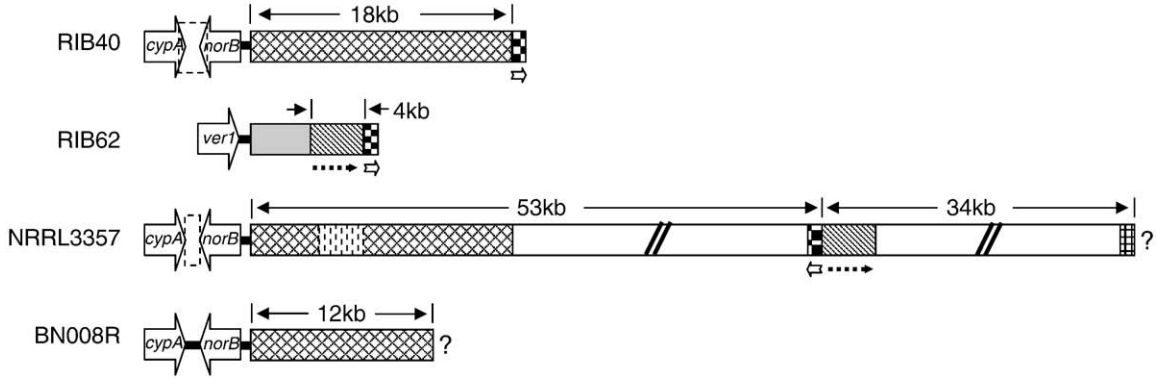

Fig. 2. Comparison of the subtelomeric regions distal to the aflatoxin gene cluster in *A. oryzae* isolates from group 1 (RIB40) and group 2 (RIB62), *A. flavus* NRRL3357, and *Aspergillus* S_{BG} strain BN008R. The open arrow indicates the location and orientation of telomeric repeat sequences. The telomeric repeat in NRRL3357 has not been located, but the terminal 1.5-kb region is TA rich. The question mark indicates a region where sequence has not been determined.

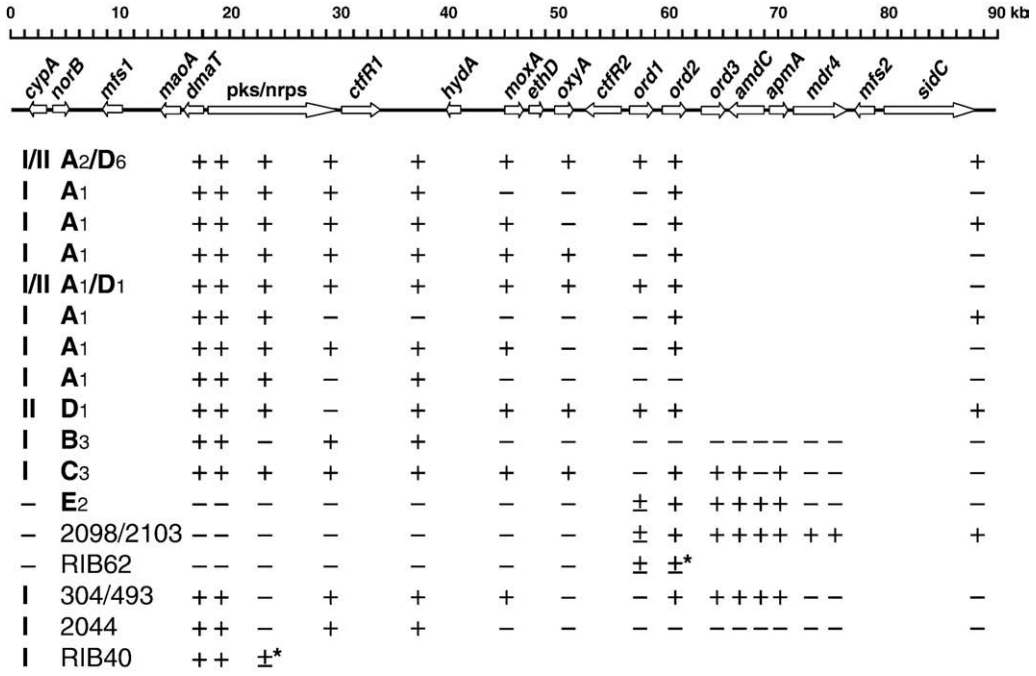

Fig. 3. PCR profiles of subtelomeric regions of *A. flavus* and *A. oryzae* strains distal to the aflatoxin cluster. The *norB* gene marks the end of the aflatoxin gene cluster. Predicted genes located in the 87-kb subtelomeric region beyond the aflatoxin gene cluster in *A. flavus* NRRL3357 are shown. I and II indicate type I and type II deletions in the *norB-cypA* region. Capital letter indicates *A. flavus* groups containing various deletions (A to E) in the aflatoxin gene cluster (see Fig. 1). The numeric indicates the number of isolates examined. Others are *A. oryzae* strains. The symbol $\pm^*$ indicates that the telomeric repeats are present in the specified region.

Carbone et al. (2007b) showed that recombination and balancing selection must have played a role in the organization of the aflatoxin gene cluster of *A. parasiticus*. Their linkage disequilibrium analyses established five distinct recombination blocks. The boundaries of recombination blocks are similar to the location of sequence breakpoints in *A. flavus* strains with partial deletion patterns (Chang et al. 2005). Loss of partial aflatoxin gene cluster in *A. oryzae* and some *A. flavus* most likely results from the release from purifying selection that could result from a long-term change in environmental niche associated with the species propagation. Alternatively, but less likely, the partial aflatoxin gene clusters may represent the ancestral states, and full clusters arose from the acquisition of gene modules from other genomic locations or via genetic exchange and recombination.

9. Vegetative incompatibility of *A. flavus* and its implication for diversity in *A. oryzae*

In fungi, vegetatively compatible hyphae can fuse to form heterokaryons and may result in fusion of different nuclei. Vegetative compatibility (VC) is determined by a series of heterokaryon incompatibility loci whose alleles have to be identical for viable hyphal fusion (Leslie, 1993). The parasexual cycle has been reported for many *Aspergillus* species (Papa, 1973, 1978). Parasexual recombination can occur during segregation of whole chromosomes as in meiosis or by mitotic crossover. VC has been used extensively to study genetic diversity of *A. flavus* populations. Variability is found among isolates from different VCGs, but there is little variability among isolates of *A. flavus* from the same VCG in either morphology, the ability to produce aflatoxins and other secondary metabolites, or the ability to infect and decay plants (Cotty et al., 1994). Although studies of VCG of *A. oryzae* have not been carried out, Ishitani and Sakaguchi (1956) showed that *A. oryzae* strains are capable of hyphal fusion. Heterokaryon incompatibility genes (*het*) and incompatibility related genes (*idi*, induced during incompatibility) that determine VC are present in the *A. oryzae* RIB40 genome (Pal et al., 2007). This suggests that the VC is (or could be) a means of generating diversity in *A. oryzae*. Wicklow et al. (2002, 2007) investigated the genotypes of *A. oryzae* strains used to produce traditional fermented foods in East Asia as well as those collected from traditional soy sauce production in Southeast Asia (Malaysia, Thailand, Singapore, and Philippines). All *A. oryzae* strains they probed with pAF28 exhibited moderate to highly repetitive profiles. Over the years, Wicklow et al. (2007) have recorded more than 70 genotypes for the *A. oryzae* strains examined. They noticed that regional differences are significant and only 8% (5 out of 64) of the *A. oryzae* isolates from Southeast Asia have RFLP patterns identical to isolates of two genotypes from East Asia (Japan, China, and Taiwan). In the studies of genetic variation of *A. flavus* isolates collected from different regions, the RFLP patterns revealed by the pAF28 probe, except for a few rare cases, consistently match with the classified VCGs. Those *A. oryzae* genotypes identified by pAF28 likely represent different VCGs. Group 1 and group 2 *A. oryzae* each appear to encompass a large number of VCGs. For example, *A. oryzae* RIB strains 23, 178, and 331 included in the aforementioned work (Wicklow et al., 2002) yielded two RFLP patterns, as do the three strains belonging to group 1 categorized by Tominaga et al. (2006).

Recombination may be prevented between species from different VCG. A comparison of *A. flavus* isolates of the same VCG separated by over 2000 km in the United States found that they had identical polymorphisms in three different genes (Ehrlich et al., 2007). Using array comparative genomic hybridization assays, Fedorova et al. (2009) showed that *A. flavus* isolates of the same VCG have almost identical gene content. In contrast, strain-specific genes comprise up to 2% of the genomes in *A. fumigatus* isolates of different VCGs. These results suggest that, at least over the time-span of the collection of these isolates, recombination is unlikely to occur. Isolates of the same VCG most probably represent a clonal assembly. Divergence time between pairs of VCG was estimated to range from 50,000 to 189,000 years ago (Grubisha and Cotty, 2009). Such divergence far preceded selection of an ancestral *A. flavus* for domestication as *A. oryzae*.

10. Cryptic recombination and mating-type genes in *A. flavus* and *A. oryzae*

In one study, aflatoxin-producing L-strain and S-strain *A. flavus* isolates were grouped into subclades (Chang et al., 2006). In the same

subclade some isolates had different sets of *omtA* gene polymorphisms from others, and at the same time, other isolates having identical *omtA* polymorphisms had different deletions in the *norB-cypA* region. The *A. oryzae* isolates included in the analysis contained exclusively type I *norB-cypA* deletion. However, one *A. oryzae* subclade (SRRC304 and SRRC493 of group 1) is phylogenetically closer to two *A. flavus* subclades in which the isolates have, respectively, type I and type II *norB-cypA* deletions. This *A. oryzae* subclade is also related to another *A. flavus* subclade having only the type II deletion. Group 1 *A. oryzae* RIB40 and SRRC2103 as well as group 2 isolates (SRRC2098 and SRRC2103) are in the same subclade as *A. flavus* containing only the type I deletion (Fig. 4). The clustered genes, *maoA-dmaT-pks/nrps*, involved in cyclopiazonic acid biosynthesis are adjacent to *norB* end of the aflatoxin gene cluster in *A. flavus* and in some *A. oryzae* strains (Tokuoka et al., 2008). Phylogeny based on the polymorphism in *dmaT*, which encodes dimethylallyl cycloacetoacetyl tryptophan synthase, shows that the aforementioned group 1 and group 2 *A. oryzae* isolates form a monophyletic assembly along with *A. flavus* isolates with the type I deletion (Fig. 5). This observed divergence for *A. oryzae* could best be explained by recombination events that caused exchanges of different regions between the two adjacent gene clusters. Although only the type I deletion in *norB-cypA* is associated with *A. oryzae*, Tominaga et al. (2006, see supplementary data) reported that a small portion (5%) of group 1 *A. oryzae* RIB strains have the type II deletion. Taken together, group 1 *A. oryzae* must have originated from ancestors common to *A. flavus* that had the entire aflatoxin gene cluster, such as the pattern A, B and D isolates of *A. flavus* previously characterized (Chang et al., 2005). Their *A. flavus* counterparts likely are still easily found in many parts of the world. Recombination and gene deletion likely occurred long before a small number of *A. oryzae* strains were selected as starter cultures for koji preparation.

In sexual heterothallic *Ascomycetes*, mating occurs between strains having complementary mating-type genes, that is *MAT1-1* (*mat1-1*, *matA*) and *MAT1-2* (*mat1-2*, *matB*). *MAT1-1* encodes a protein containing a α-box domain, and *MAT1-2* encodes a protein containing a high mobility group (HMG) type DNA-binding domain (Turgeon and Yoder, 2000). The mating-type proteins appear to be functionally conserved. When the *A. fumigatus mat1-2* open reading frame was driven by the *A. nidulans matA* promoter, it conferred full fertility (Pyrzak et al., 2008). Species in *Aspergillus* section *Flavi* have long been considered strictly anamorphic. Genome information about *A. flavus* NRRL3357 and *A. oryzae* RIB40 has revolutionized the current thinking about sexual reproduction in these species. *MAT1-1* is present in the *A. flavus* NRRL3357 genome (AFL2G_11189.2; http://www.broad.mit.edu/annotation/genome/aspergillus_group/GenomesIndex.html) and is also present on chromosome 6 of the *A. oryzae* RIB40 genome (AO090020000089; http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao), which indicates a heterothallic organization of the mating-type genes in these species. Ramirez-Prado et al. (2008) isolated the *MAT1-2* gene of *A. flavus* and *MAT1-1* and *MAT1-2* genes of the closely related *A. parasiticus*. They studied *A. flavus* and *A. parasiticus* populations from a peanut field in Georgia. Of the isolates analyzed 15 *A. flavus* isolates had *MAT1-1* and 58 had *MAT1-2* and 62 *A. parasiticus* strains had *MAT1-1* and 14 had *MAT1-2*, alleles. By crossing strains having *MAT1-1* with those having *MAT1-2* they were able to demonstrate a sexual stage in *A. flavus* and *A. parasiticus* (Horn et al., 2009a,b). In *A. parasiticus* the aflatoxin gene cluster and a subtelomeric region on chromosome 3 and the mating type genes on chromosome 6 were found to segregate independently based on the detection of specific haplotypes in the progeny. Like *A. flavus* and *A. parasiticus*, an *A. oryzae* strain with the type I deletion has either *MAT1-1* or *MAT1-2* (Fig. 6). Different mating-type genes are also found in S-strain *A. flavus* isolates. As mentioned earlier, group 2 *A. oryzae* SRRC2098 and SRRC2103 have the same breakpoint upstream of *ver1* as the pattern E *A. flavus* isolates (Chang et al., 2006), but the two *A. oryzae* isolates have different mating-type genes. *A. oryzae* RIB40 and SRRC 2044 belong to the same clade based on *omtA* and *dmaT* polymorphisms (Figs. 4 and 5), but they have different mating-type genes. Because aflatoxin and cyclopiazonic acid gene clusters reside in the chromosome 3 subtelomeric region and the mating genes are on chromosome 6, it strongly suggests that sexual recombination must have occurred in *A. oryzae* ancestors and generated the extant isolates. *A. oryzae* strains predominantly have type I *norB-cypA* deletion; it suggests that ancestral S-strain derivatives that eventually evolved to nonaflatoxigenic L-strain and selected as *A. oryzae* had the most suitable characteristics for use in food fermentation.

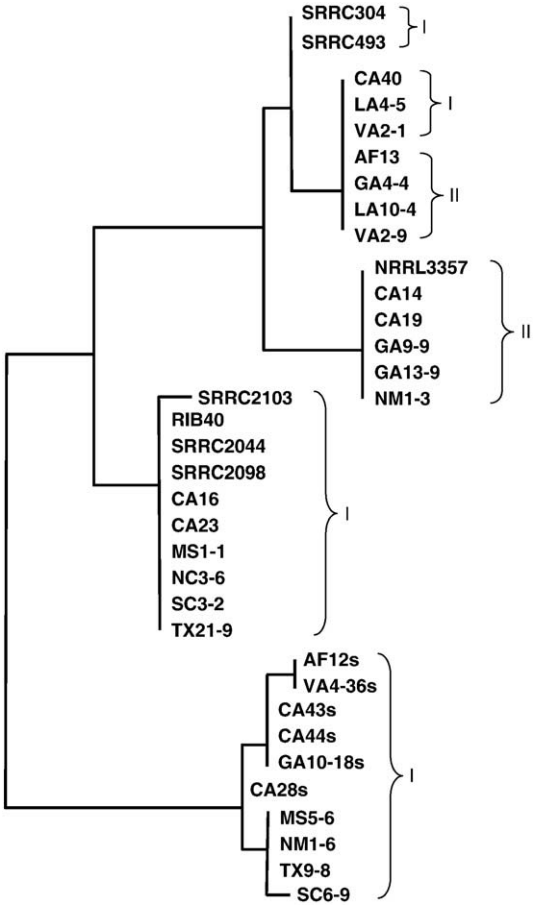

Fig. 4. Phylogenetic tree showing relationships of *A. flavus* and *A. oryzae* isolates based on the single nucleotide polymorphisms of aflatoxin biosynthesis *omtA* genes. RIB and SRRC (Southern Regional Research Center) strains are *A. oryzae*, and others are *A. flavus*. I and II indicate the deletion pattern in the *norB-cypA* region.

11. Variants of *A. flavus* and *A. oryzae*

Natural variants are known to be present in the populations of each fungal species. *A. flavus* isolates vary considerably in their abilities to produce the toxic metabolites of aflatoxins and cyclopiazonic acid and to colonize plants. *A. oryzae* strains have never been reported to produce aflatoxins but some strains produce cyclopiazonic acid. Besides the aforementioned S- and L-strain aflatoxigenic morphotypes there are *A. flavus* variants which are incapable of aflatoxin production (Horn and Dorner, 1999). No *A. oryzae* isolates that produce small sclerotia have been reported. The mycelium of *A. oryzae* is floccose with sparse sporulation. Aging colonies of *A. oryzae* tend to change to brown color, but *A. flavus* colonies typically retain the yellowish-greenish color. Wild-type *A. flavus* isolates often degenerate after serial transfers on culture media (Horn and Dorner,

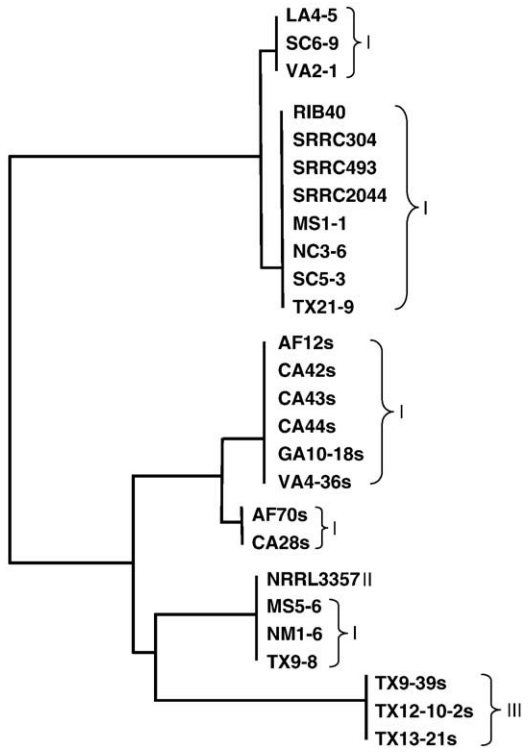

Fig. 5. Phylogenetic tree showing relationships of *A. flavus* and *A. oryzae* isolates based on the single nucleotide polymorphisms of *dmaT* genes involved in cyclopiazonic acid biosynthesis.

2002). Degeneration is accompanied by morphological changes such as increased floccose growth, decreased sporulation and sclerotial production, and a shift in conidial color from green to brown, characteristics reminiscent of *A. oryzae* isolates (Torres et al., 1980; Bilgrami et al., 1988). Strain degeneration in *A. parasiticus* can be artificially induced by transfer of macerated mycelia without allowing conidiation (Kale et al., 2003) or by treatment of fungal cells with azacytidine, an agent that inhibits DNA-dependent cytosine methyltransferases (Tamame et al., 1983; Gabbara and Bhagwat, 1995). Strain degeneration is irreversible. Genome rearrangements, such as amplifications and deletions, are regarded as responses to strong, sustained selective pressure of the growth environments on the microbial populations. In *Saccharomyces*, experimentally induced genome rearrangements were found to be bounded by transposon-related sequences at the DNA breakpoints (Dunham et al., 2002). Associated genetic alterations in degenerated *A. flavus* could be the result of DNA rearrangements due to activation of mobile genetic

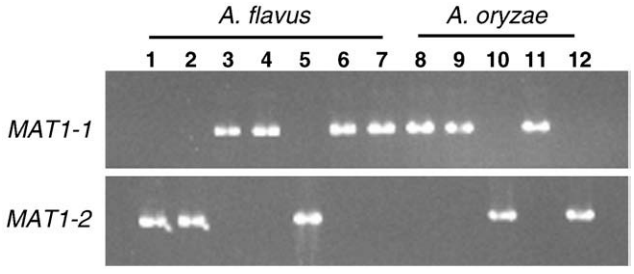

Fig. 6. Mating-type genes in S-strain *A. flavus* and *A. oryzae* isolates. Lanes 1, CA28; 2, CA42; 3, CA43; 4, CA44; 5, AF12s, 6, GA10-18s; 7, VA4-36s; 8, SRRC304; 9, SRRC493; 10, SRRC2044; 11, SRRC2098 and 12, SRRC2103. The mating-type specific oligonucleotides were derived from *A. flavus MAT1-2* (GenBank accession number: EU357934) and *MAT2-2* (EU357936). MAT1-1F: atggaaaccacagtgtctcc, MAT1-1R: tcaacgaatctagagaagtc amplify a 1161-bp region. MAT1-2F: atcgagaatgacgactatac and MAT1-2R: ttcttcagtag-cagtcagca amplify a 1061-bp region.

elements. *A. oryzae* OSI1013 had multiple intact copies of the transposon *Crawler* as mentioned above, but *A. oryzae* RIB40 has only one degenerate copy of the transposon element in the genome. *A. oryzae* RIB40 has many copies of the transposon element *Tao1* (GenBank accession number: AB021710) including those with various degrees of degeneracy. NRRL3357 has a much lower copy number of *Tao1* and does not harbor *Crawler* or the retrotransposon *AFLAV*. While transcription of *Crawler* occurred under standard growth conditions, transcription increased in the presence of stress-inducing treatments and, concomitantly, transposition (Ogasawara et al., 2009). *Tao1* and *AFLAV* may undergo similar changes in activity when subject to stress either in the field or in the laboratory. The finding that the strain chosen for genome sequencing, *A. flavus* NRRL3357, appeared to harbor less of the mobile elements is intriguing. A clue might lie in the evolutionary history. The aflatoxigenic L-strain *A. flavus* with the type II deletion, such as NRRL3357 and others (Chang et al., 2009), which still retain cyclopiazonic acid production, probably have fewer genome aberrations, such as rearrangements, translocations, and gene deletions than atoxigenic group 1 *A. oryzae*, such as RIB40 which has the type I S-strain signature deletion in its *norB-cypA* region. As mentioned earlier, the *Crawler* transposon of RIB40 (AB256519) shares only 90% nucleotide identity to that in group 2 *A. oryzae* RIB128 (AB256517) and group 4 *A. oryzae* RIB209 (AB256518). In contrast, the intact *Crawler* of OSI1013 (AB256520) shares 100% and >99% nucleotide identity to those of RIB128 and RIB209, respectively. The overall activity of the mobile elements in the genome could be significant to the evolution of *A. oryzae* and *A. flavus* as distinct variants.

12. Evolution of *A. flavus* and *A. oryzae*

A. oryzae has an increased number of genes encoding hydrolytic enzymes than the other sequenced aspergilli. It also possesses more secretory acidic proteinase genes, a fact that may reflect an increased ability to adapt to acidic environments (Machida et al., 2005). The increase in the size of the *A. oryzae* genome relative to *A. fumigatus* and *A. nidulans* does not appear to be due to chromosomal duplication because no extended stretches of duplicated regions were found (Galagan et al., 2005). Speciation of *A. flavus* has been estimated to have taken place approximately 5–8 million years ago (Ehrlich, 2008). *A. oryzae* has substantially more proteins involved in primary and secondary metabolism than *A. fumigatus* and *A. nidulans*. Virtually the same gene complement is found in *A. flavus* NRRL3357, an isolate presumably not associated with domestication (Rokas, 2009). This suggests that genome expansion in *A. oryzae* must have preceded its domestication.

The main differences between *A. oryzae* and *A. flavus* are associated predominantly with developmental traits, including the lack of secondary metabolism. Nonaflatoxigenic *A. flavus* isolates are widespread in nature and in some cases grouped in the same clade with *A. oryzae* isolates but in other cases form separate groupings. At this point we can only speculate about the distinction between *A. flavus* and *A. oryzae* that has made the latter a commercially useful organism while the former is a nemesis to worldwide agriculture. *A. flavus* has developed an extraordinary ability among *Aspergillus* species to colonize plants (Cotty et al., 1994). This ability to escape the normally saprophytic role associated with related aflatoxin-producing *A. nomius* and *A. parasiticus* could have resulted from the gene expansion described above: the increased contents of proteolytic enzyme-encoding genes, nitrogen utilization genes and genes involved in carbohydrate metabolism. This expansion clearly is associated with a change in the lifestyle of the fungus. Such changes may have been adaptations to a new living environment, possibly an adaptation to the emergence of grasslands during interglacial periods when regions of the earth became more temperate and where *A. flavus* had been living mainly as a saprophyte. Grasslands became widespread both in

North America and Africa at the expected time of divergence (about 5–8 Mya) of *A. flavus* from an ancestral B and G-producing species (Stromberg, 2005; Osborne, 2008). Grasslands are thought to be maintained by dry periods that allow frequent lightning-ignited fires (Anderson, 2006). Fires would increase the N/C ratio in the ash. This altered nutrient composition is in accord with the increases in nitrogen-utilizing genes found in *A. flavus*. Eventually agriculture was developed, and with the loss of selection pressure for toxin and secondary metabolite production, nonaflatoxigenic strains of *A. flavus* may have become a common variant in the population. So common, that depending on the agricultural environments tested, 30% and up to 80% of the isolates of *A. flavus* from a particular region may be nonaflatoxigenic (Guzman and Sanchez, 1994; Horn and Dorner, 1999; Vaamonde et al., 2003; Pildain et al., 2004; Razzaghi-Abyaneh et al., 2006; Sanchez-Hervas et al., 2008; Yin et al., 2009). We hypothesize that it is from this pool of already existing nonaflatoxigenic *A. flavus* isolates that *A. oryzae* isolates used in food fermentations were selected.

13. Concluding remarks

Studies of the genome sequences and phylogeny of *A. flavus* and *A. oryzae* have provided insight into the genetic similarity between and diversity within these two economically important species. With this insight we redefine previously understood concepts about the origin and evolution of the *A. oryzae* groups. From genome analysis, we and others conclude that *A. oryzae* is not merely a domesticated *A. flavus.* The genome expansion in *A. flavus* and *A. oryzae* compared to other *Aspergillus* species is an intriguing issue that we have postulated arises from a change in adaptation. The development of rapid genome sequencing techniques will soon make many *Aspergillus* genome sequences available which will enable a comprehensive analysis of the role of recombination and gene duplication in fostering genetic diversity within *A. flavus* and *A. oryzae.* Such studies should allow us to determine if interactions with agriculture affects the evolution of *A. flavus* and *A. oryzae*. A predominantly saprophytic *A. flavus* ancestor likely became increasingly dependent on associations with plants. If our hypothesis about the origin of *A. flavus* and *A. oryzae* is correct, certain genetic traits common to plant epiphytes then may be present in *A. flavus* but not in strictly saprophytic fungi. This understanding could affect the decisions currently being made about which atoxigenic *Aspergillus* will be the best competitor strains to reduce crop contamination with aflatoxin. In addition, new sources of useful fungi for fermentation may be developed from the large pool of atoxigenic *A. flavus* isolated from plants. Both of these outcomes are felicitous consequences of improved understanding of the intersect between *A. flavus* and *A. oryzae*.

References

Anderson, R.C., 2006. Evolution and origin of the Central Grassland of North America: climate, fire, and mammalian grazers. Journal of the Torrey Botanical Society 133, 626–647.

Barbesgaard, P., Heldt-Hansen, H.P., Diderichsen, B., 1992. On the safety of *Aspergillus oryzae*: a review. Applied Microbiology and Biotechnology 36, 569–572.

Bennett, J.W., 1985. Molds, Manufacturing and Molecular Genetics. In: Timberlake, W.E. (Ed.), Molecular Genetics of Filamentous Fungi. Alan R. Liss, Inc., New York, pp. 345–366.

Bhattacharyya, A., Blackburn, E.H., 1997. *Aspergillus nidulans* maintains short telomeres throughout development. Nucleic Acids Research 25, 1426–1431.

Bigelis, R., 1992. Food enzymes. In: Finkelstein, D.B., Ball, C. (Eds.), Biotechnology of Filamentous Fungi: Technology and Product. Butterworth-Heinemann, Boston, Massachusetts, pp. 361–415.

Bilgrami, K.S., Sinha, S.P., Jeswal, P., 1988. Loss of toxigenicity of *Aspergillus flavus* strains during subculturing-a genetic interpretation. Current Science 57, 551–552.

Carbone, I., Jakobek, J.L., Ramirez-Prado, J.H., Horn, B.W., 2007a. Recombination, balancing selection and adaptive evolution in the aflatoxin gene cluster of *Aspergillus parasiticus*. Molecular Ecology 16, 4401–4417.

Carbone, I., Ramirez-Prado, J.H., Jakobek, J.L., Horn, B.W., 2007b. Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. BMC Evolutionary Biology 7, 111.

Cary, J.W., Wright, M., Bhatnagar, D., Lee, R., Chu, F.S., 1996. Molecular characterization of an *Aspergillus parasiticus* dehydrogenase gene, *norA*, located on the aflatoxin biosynthesis gene cluster. Applied and Environmental Microbiology 62, 360–366.

Cary, J.W., Ehrlich, K.C., Bland, J.M., Montalbano, B.G., 2006. The aflatoxin biosynthesis cluster gene, *aflX*, encodes an oxidoreductase involved in conversion of versicolorin A to demethylsterigmatocystin. Applied and Environmental Microbiology 72, 1096–1101.

Cary, J.W., Ehrlich, K.C., Beltz, S.B., Harris-Coward, P., Klich, M.A., 2009. Characterization of the *Aspergillus ochraceoroseus* aflatoxin/sterigmatocystin biosynthetic gene cluster. Mycologia 101, 352–362.

Chang, P.-K., 2003. The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. Molecular Genetics and Genomics 268, 711–719.

Chang, P.-K., Horn, B.W., Dorner, J.W., 2005. Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. Fungal Genetics and Biology 42, 914–923.

Chang, P.-K., Ehrlich, K.C., Hua, S.S., 2006. Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates. International Journal of Food Microbiology 108, 172–177.

Chang, P.-K., Horn, B.W., Dorner, J.W., 2009. Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. Fungal Genetics and Biology 46, 176–182.

Coleman, M.J., McHale, M.T., Arnau, J., Watson, A., Oliver, R.P., 1993. Cloning and characterisation of telomeric DNA from *Cladosporium fulvum*. Gene 132, 67–73.

Cotty, P.J., 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79, 808–814.

Cotty, P.J., Bayman, D.S., Egel, D.S., Elias, K.S., 1994. Agriculture, Aflatoxins and *Aspergillus*. In: Powell, K. (Ed.), The Genus *Aspergillus*. Plenum Press, New York, pp. 1–27.

Criseo, G., Bagnara, A., Bisignano, G., 2001. Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. Letters in Applied Microbiology 33, 291–295.

Dunham, M.J., Badrane, H., Ferea, T., Adams, J., Brown, P.O., Rosenzweig, F., Botstein, D., 2002. Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United States of America 99, 16144–16149.

Ehrlich, K.C., 2008. Genetic diversity in *Aspergillus flavus* and its implications for agriculture. In: Varga, J., Samson, R.A. (Eds.), *Aspergillus* in the genomics era. Wageningen Academic Publishers, Wageningen, pp. 233–247.

Ehrlich, K.C., Chang, P.-K., Yu, J., Cotty, P.J., 2004. Aflatoxin biosynthesis cluster gene *cypA* is required for G aflatoxin formation. Applied and Environmental Microbiology 70, 6518–6524.

Ehrlich, K.C., Montalbano, B.G., Cotty, P.J., 2007. Analysis of single nucleotide polymorphisms in three genes shows evidence for genetic isolation of certain *Aspergillus flavus* vegetative compatibility groups. FEMS Microbiology Letters 268, 231–236.

Farman, M.L., Leong, S.A., 1995. Genetic and physical mapping of telomeres in the rice blast fungus, *Magnaporthe grisea*. Genetics 140, 479–492.

Fedorova, N.D., Harris, S., Chen, D., Denning, D.W., Yu, J., Cotty, P.J., Nierman, W.C., 2009. Using aCGH to study intraspecific genetic variability in two pathogenic molds, *Aspergillus fumigatus* and *Aspergillus flavus*. Medical Mycology 47 (Suppl), 1–8.

Frisvad, J.C., Skouboe, P., Samson, R.A., 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B_1, sterigmatocystin and 3-*O*-methylsterigmatocystin, *Aspergillus rambellii sp. nov*. Systematic and Applied Microbiology 28, 442–453.

Fuchs, J., Brandes, A., Schubert, I., 1995. Telomere sequence localization and karyotype evolution in higher plants. Plant Systematics and Evolution 196, 227–241.

Gabbara, S., Bhagwat, A.S., 1995. The mechanism of inhibition of DNA (cytosine-5-)-methyltransferases by 5-azacytosine is likely to involve methyl transfer to the inhibitor. Biochemcal Journal 307, 87–92.

Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.J., Wortman, J.R., Batzoglou, S., Lee, S.I., Basturkmen, M., Spevak, C.C., Clutterbuck, J., Kapitonov, V., Jurka, J., Scazzocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G.H., Draht, O., Busch, S., D'Enfert, C., Bouchier, C., Goldman, G.H., Bell-Pedersen, D., Griffiths-Jones, S., Doonan, J.H., Yu, J., Vienken, K., Pain, A., Freitag, M., Selker, E.U., Archer, D.B., Penalva, M.A., Oakley, B.R., Momany, M., Tanaka, T., Kumagai, T., Asai, K., Machida, M., Nierman, W.C., Denning, D.W., Caddick, M., Hynes, M., Paoletti, M., Fischer, R., Miller, B., Dyer, P., Sachs, M.S., Osmani, S.A., Birren, B.W., 2005. Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature 438, 1105–1115.

Geiser, D.M., Pitt, J.I., Taylor, J.W., 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proceedings of the National Academy of Sciences of the United States of America 95, 388–393.

Geiser, D.M., Dorner, J.W., Horn, B.W., Taylor, J.W., 2000. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. Fungal Genetics and Biology 31, 169–179.

Georgianna, D.R., Payne, G.A., 2009. Genetic regulation of aflatoxin biosynthesis: from gene to genome. Fungal Genetics and Biology 46, 113–125.

Grubisha, L.C., Cotty, P.J., 2009. Parasexual recombination and migration maintain genotypic diversity in the aflatoxin-producing fungal plant pathogen *Aspergillus flavus*. The American Phytopathological Society Annual Meeting, Portland, Oregon, p. S47. August 1–5.

Guzman, P.A., Sanchez, J.G., 1994. Characterization of telomeric regions from *Ustilago maydis*. Microbiology 140, 551–557.

Hijri, M., Niculita, H., Sanders, I.R., 2007. Molecular characterization of chromosome termini of the arbuscular mycorrhizal fungus *Glomus intraradices* (Glomeromycota). Fungal Genetics and Biology 44, 1380–1386.

Horn, B.W., Dorner, J.W., 1999. Regional differences in production of aflatoxin B_1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Applied and Environmental Microbiology 65, 1444–1449.

Horn, B.W., Dorner, J.W., 2002. Effect of competition and adverse culture conditions on aflatoxin production by *Aspergillus flavus* through successive generations. Mycologia 94, 741–751.
Horn, B.W., Moore, G.G., Carbone, I., 2009a. Sexual reproduction in *Aspergillus flavus*. Mycologia 101, 423–429.
Horn, B.W., Ramirez-Prado, J.H., Carbone, I., 2009b. Sexual reproduction and recombination in the aflatoxin-producing fungus *Aspergillus parasiticus*. Fungal Genetics and Biology 46, 169–175.
Hua, S.S., Tarun, A.S., Pandey, S.N., Chang, L., Chang, P.-K., 2007. Characterization of *AFLAV*, a *Tf1/Sushi* retrotransposon from *Aspergillus flavus*. Mycopathologia 163, 97–104.
Ishitani, C., Ikeda, Y., Sakaguchi, K., 1956. Hereditary variation and recombination in koji-molds (*Aspergillus oryzae* and *Asp. sojae*). V. Heterocaryosis. The Journal of General and Applied Microbiology 2, 345–400.
James, M.J., Lasker, B.A., McNeil, M.M., Shelton, M., Warnock, D.W., Reiss, E., 2000. Use of a repetitive DNA probe to type clinical and environmental isolates of *Aspergillus flavus* from a cluster of cutaneous infections in a neonatal intensive care unit. Journal of Clinical Microbiology 38, 3612–3618.
Javerzat, J.P., Bhattacherjee, V., Barreau, C., 1993. Isolation of telomeric DNA from the filamentous fungus *Podospora anserina* and construction of a self-replicating linear plasmid showing high transformation frequency. Nucleic Acids Research 21, 497–504.
Jiang, J., Yan, L., Ma, Z., 2009. Molecular characterization of an atoxigenic *Aspergillus flavus* strain AF051. Applied Microbiology and Biotechnology 83, 501–505.
Jorgensen, T.R., 2007. Identification and toxigenic potential of the industrially important fungi, *Aspergillus oryzae* and *Aspergillus sojae*. Journal of Food Protection 70, 2916–2934.
Kale, S.P., Cary, J.W., Baker, C., Walker, D., Bhatnagar, D., Bennett, J.W., 2003. Genetic analysis of morphological variants of *Aspergillus parasiticus* deficient in secondary metabolite production. Mycological Research 107, 831–840.
Khaldi, N., Wolfe, K.H., 2008. Elusive origins of the extra genes in *Aspergillus oryzae*. PLoS ONE 3, e3036.
Klich, M.A., Mullaney, E.J., 1987. DNA restriction enzyme fragment polymorphism as a tool for rapid differentiation of *Aspergillus flavus* from *Aspergillus oryzae*. Experimental Mycology 1, 170–175.
Klich, M.A., Yu, J., Chang, P.K., Mullaney, E.J., Bhatnagar, D., Cleveland, T.E., 1995. Hybridization of genes involved in aflatoxin biosynthesis to DNA of aflatoxigenic and non-aflatoxigenic aspergilli. Applied Microbiology and Biotechnology 44, 439–443.
Krishnan, S., Manavathu, E.K., Chandrasekar, P.H., 2009. *Aspergillus flavus*: an emerging non-fumigatus *Aspergillus* species of significance. Mycoses 52, 206–222.
Kumeda, Y., Asao, T., 2001. Heteroduplex panel analysis, a novel method for genetic identification of *Aspergillus* section *Flavi* strains. Applied and Environmental Microbiology 67, 4084–4090.
Kurtzman, C.P., Smiley, M.J., Robert, C.J., Wicklow, D.T., 1986. DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. Mycologia 78, 955–959.
Kusumoto, K., Nogata, Y., Ohta, H., 2000. Directed deletions in the aflatoxin biosynthesis gene homolog cluster of *Aspergillus oryzae*. Current Genetics 37, 104–111.
Kusumoto, K.I., Suzuki, S., Kashiwagi, Y., 2003. Telomeric repeat sequence of *Aspergillus oryzae* consists of dodeca-nucleotides. Applied Microbiology and Biotechnology 61, 247–251.
Lee, C.Z., Liou, G.Y., Yuan, G.F., 2006a. Comparison of the *aflR* gene sequences of strains in *Aspergillus* section *Flavi*. Microbiology 152, 161–170.
Lee, K.W., Lee, S.K., Lee, B.D., 2006b. *Aspergillus oryzae* as probiotic in poultry: a review. International Journal of Poultry Science 5, 1–3.
Lee, Y.H., Tominaga, M., Hayashi, R., Sakamoto, K., Yamada, O., Akita, O., 2006c. *Aspergillus oryzae* strains with a large deletion of the aflatoxin biosynthetic homologous gene cluster differentiated by chromosomal breakage. Applied Microbiology and Biotechnology 72, 339–345.
Leslie, J.F., 1993. Fungal vegetative compatibility. Annual review of Phytopathology 31, 127–150.
Levis, C., Giraud, T., Dutertre, M., Fortini, D., Brygoo, Y., 1997. Telomeric DNA of *Botrytis cinerea*: a useful tool for strain identification. FEMS Microbiology Letters 157, 267–272.
Long, D.M., Smidansky, E.D., Archer, A.J., Strobel, G.A., 1998. In vivo addition of telomeric repeats to foreign DNA generates extrachromosomal DNAs in the taxol-producing fungus *Pestalotiopsis microspora*. Fungal Genetics and Biology 24, 335–344.
Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., Abe, K., Gomi, K., Horiuchi, H., Kitamoto, K., Kobayashi, T., Takeuchi, M., Denning, D.W., Galagan, J.E., Nierman, W.C., Yu, J., Archer, D.B., Bennett, J.W., Bhatnagar, D., Cleveland, T.E., Fedorova, N.D., Gotoh, O., Horikawa, H., Hosoyama, A., Ichinomiya, M., Igarashi, R., Iwashita, K., Juvvadi, P.R., Kato, M., Kato, Y., Kin, T., Kokubun, A., Maeda, H., Maeyama, N., Maruyama, J., Nagasaki, H., Nakajima, T., Oda, K., Okada, K., Paulsen, I., Sakamoto, K., Sawano, T., Takahashi, M., Takase, K., Terabayashi, Y., Wortman, J.R., Yamada, O., Yamagata, Y., Anazawa, H., Hata, Y., Koide, Y., Komori, T., Koyama, Y., Minetoki, T., Suharnan, S., Tanaka, A., Isono, K., Kuhara, S., Ogasawara, N., Kikuchi, H., 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature 438, 1157–1161.
Machida, M., Yamada, O., Gomi, K., 2008. Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future. DNA Research 15, 173–183.
McAlpin, C.E., Mannarelli, B., 1995. Construction and characterization of a DNA probe for distinguishing strains of *Aspergillus flavus*. Applied and Environmental Microbiology 61, 1068–1072.
McAlpin, C.E., Wicklow, D.T., Horn, B.W., 2002. DNA fingerprinting analysis of vegetative compatibility groups in *Aspergillus flavus* from a peanut field in Georgia. Plant Disease 86, 254–258.
Montiel, D., Dickinson, M.J., Lee, H.A., Dyer, P.S., Jeenes, D.J., Roberts, I.N., James, S., Fuller, L.J., Matsuchima, K., Archer, D.B., 2003. Genetic differentiation of the *Aspergillus* section *Flavi* complex using AFLP fingerprints. Mycological Research 107, 1427–1434.
Nikkuni, S., Nakajima, H., Hoshina, S., Ohno, M., Suzuki, C., Kashiwagi, Y., Mori, K., 1998. Evolutionary relationships among *Aspergillus oryzae* and related species based on the sequences of 18S rRNA genes and internal transcribed spacers. The Journal of General and Applied Microbiology 44, 225–230.
Ogasawara, H., Obata, H., Hata, Y., Takahashi, S., Gomi, K., 2009. *Crawler*, a novel *Tc1/mariner*-type transposable element in *Aspergillus oryzae* transposes under stress conditions. Fungal Genetics and Biology 46, 441–449.
Okubara, P.A., Tibbot, B.K., Tarun, A.S., McAlpin, C.E., Hua, S.S., 2003. Partial retrotransposon-like DNA sequence in the genomic clone of *Aspergillus flavus*, pAF28. Mycological Research 107, 841–846.
Orum, T.V., Bigelow, D.M., Nelson, M.R., Howell, D.R., Cotty, P.J., 1997. Spatial and temporal patterns of *Aspergillus flavus* strain composition and propagule density in Yuma County, Arizona soils. Plant Disease 81, 911–916.
Osborne, C.P., 2008. Atmosphere, ecology and evolution: what drove the Miocene expansion of C(4) grasslands? Journal of Ecology 96, 35–45.
Pal, K., van Diepeningen, A.D., Varga, J., Hoekstra, R.F., Dyer, P.S., Debets, A.J., 2007. Sexual and vegetative compatibility genes in the aspergilli. Studies in Mycology 59, 19–30.
Papa, K.E., 1973. The parasexual cycle in *Aspergillus flavus*. Mycologia 65, 1201–1205.
Papa, K.E., 1978. The parasexual cycle in *Aspergillus parasiticus*. Mycologia 70, 766–773.
Payne, G.A., Nierman, W.C., Wortman, J.R., Pritchard, B.L., Brown, D., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Machida, M., Yu, J., 2006. Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. Medical Mycology 44 (Suppl), 9–11.
Pildain, M.B., Vaamonde, G., Cabral, D., 2004. Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. International Journal of Food Microbiology 93, 31–40.
Pildain, M.B., Frisvad, J.C., Vaamonde, G., Cabral, D., Varga, J., Samson, R.A., 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. International Journal of Systematic and Evolutionary Microbiology 58, 725–735.
Powell, W.A., Kistler, H.C., 1990. In vivo rearrangement of foreign DNA by *Fusarium oxysporum* produces linear self-replicating plasmids. Journal of Bacteriology 172, 3163–3171.
Pyrzak, W., Miller, K.Y., Miller, B.L., 2008. Mating type protein Mat1-2 from asexual *Aspergillus fumigatus* drives sexual reproduction in fertile *Aspergillus nidulans*. Eukaryotic Cell 7, 1029–1040.
Ramirez-Prado, J.H., Moore, G.G., Horn, B.W., Carbone, I., 2008. Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genetics and Biology 45, 1292–1299.
Raper, K.B., Fennell, D.I., 1965. The Genus *Aspergillus*. Williams & Wilkins Company, Baltimore.
Razzaghi-Abyaneh, M., Shams-Ghahfarokhi, M., Allameh, A., Kazeroon-Shiri, A., Ranjbar-Bahadori, S., Mirzahoseini, H., Rezaee, M.B., 2006. A survey on distribution of *Aspergillus* section *Flavi* in corn field soils in Iran: population patterns based on aflatoxins, cyclopiazonic acid and sclerotia production. Mycopathologia 161, 183–192.
Rokas, A., 2009. The effect of domestication on the fungal proteome. Trends in Genetics 25, 60–63.
Rokas, A., Payne, G., Fedorova, N.D., Baker, S.E., Machida, M., Yu, J., Georgianna, D.R., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Wortman, J.R., Maiti, R., Joardar, V., Amedeo, P., Denning, D.W., Nierman, W.C., 2007. What can comparative genomics tell us about species concepts in the genus *Aspergillus*? Studies in Mycology 59, 11–17.
Saito, M., Tsuruta, O., 1993. A new variety of *Aspergillus flavus* from tropical soil in Thailand and its aflatoxin productivity. Proceedings of Japanese Association of Mycotoxicology 37, 31–36.
Sanchez-Hervas, M., Gil, J.V., Bisbal, F., Ramon, D., Martinez-Culebras, P.V., 2008. Mycobiota and mycotoxin producing fungi from cocoa beans. International Journal of Food Microbiology 125, 336–340.
Schechtman, M.G., 1990. Characterization of telomere DNA from *Neurospora crassa*. Gene 88, 159–165.
Scheidegger, K.A., Payne, G.A., 2003. Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. Toxin Reviews 22, 423–459.
Stromberg, C.A., 2005. Decoupled taxonomic radiation and ecological expansion of open-habitat grasses in the Cenozoic of North America. Proceedings of the National Academy of Sciences of the United States of America 102, 11980–11984.
Tamame, M., Antequera, F., Villanueva, J.R., Santos, T., 1983. High-frequency conversion to a "fluffy" developmental phenotype in *Aspergillus* spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. Molecular and Cellular Biology 3, 2287–2297.
Tokuoka, M., Seshime, Y., Fujii, I., Kitamoto, K., Takahashi, T., Koyama, Y., 2008. Identification of a novel polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) gene required for the biosynthesis of cyclopiazonic acid in *Aspergillus oryzae*. Fungal Genetics and Biology 45, 1608–1615.
Tominaga, M., Lee, Y.H., Hayashi, R., Suzuki, Y., Yamada, O., Sakamoto, K., Gotoh, K., Akita, O., 2006. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB strains. Applied and Environmental Microbiology 72, 484–490.
Torres, J., Guarro, J., Suarez, G., Sune, N., Calva, M.A., Ramirez, C., 1980. Morphological changes in strains of *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare related with aflatoxin production. Mycopathologia 72, 171–174.

Turgeon, B.G., Yoder, O.C., 2000. Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genetics and Biology 31, 1–5.

Underwood, A.P., Louis, E.J., Borts, R.H., Stringer, J.R., Wakefield, A.E., 1996. *Pneumocystis carinii* telomere repeats are composed of TTAGGG and the subtelomeric sequence contains a gene encoding the major surface glycoprotein. Molecular Microbiology 19, 273–281.

Vaamonde, G., Patriarca, A., Fernandez Pinto, V., Comerio, R., Degrossi, C., 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. International Journal of Food Microbiology 88, 79–84.

Vitkova, M., Kral, J., Traut, W., Zrzavy, J., Marec, F., 2005. The evolutionary origin of insect telomeric repeats, (TTAGG)n. Chromosome Research 13, 145–156.

Watanabe, C.M., Townsend, C.A., 2002. Initial characterization of a type I fatty acid synthase and polyketide synthase multienzyme complex NorS in the biosynthesis of aflatoxin B_1. Chemistry and Biology 9, 981–988.

Wicklow, D.T., McAlpin, C.E., Platis, C.E., 1998. Characterization of the *Aspergillus flavus* population within an Illinois maize field. Mycological Research 102, 263–268.

Wicklow, D.T., McAlpin, C.E., Peterson, S.W., 2002. Common genotypes (RFLP) within a diverse collection of yellow-green aspergilli used to produce traditional Oriental fermented foods. Mycoscience 43, 289–297.

Wicklow, D.T., McAlpin, C.E., Yeoh, Q.L., 2007. Diversity of *Aspergillus oryzae* genotypes (RFLP) isolated from traditional soy sauce production within Malaysia and Southeast Asia. Mycoscience 48, 373–380.

Yin, Y., Lou, T., Yan, L., Michailides, T.J., Ma, Z., 2009. Molecular characterization of toxigenic and atoxigenic *Aspergillus flavus* isolates, collected from peanut fields in China. Journal of Applied Microbiology 107, 1857–1865.

Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P., Bennett, J.W., 2004. Clustered pathway genes in aflatoxin biosynthesis. Applied and Environmental Microbiology 70, 1253–1262.